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| 13. ABSTRACT (Maximum 200 Words) Environmental factors such as sedentary life-style and high fat diet have been associated with an increase in prostate cancer risk. An explanation of the linkage between the environmental factors and prostate cancer risk might be the influence of these factors on hormone metabolism, in particular estrogen metabolism. For instance, there is experimental evidence showing that a sedentary life-style and high fat diet induce estrogen metabolism toward 16 α hydroxylation leading to biologically potent metabolites (estriol and 16 α hydroxyestrone). An active life-style and a low fat diet induce the alternative 2-hydroxylation with production of weak estrogen metabolites (2-hydroxyestrone). Potent estrogens may increase prostate cell division and increase prostate cancer risk. The research hypothesis is that the preferential induction of the 16 α hydroxylation pathway in respect to the 2-hydroxylation, is associated with an increase risk of prostate cancer. To test this hypothesis a population based case-control study is conducted. Men age 50-79, African American and white, residents in Erie and Niagara Counties in Western New York, with incident, pathologically confirmed prostate cancer are interviewed. Controls are interviewed as part of other funded on-going case-control studies and randomly selected from the general population. Urine is used for the determinations of the 16 α and 2-hydroxyestrone. Diet, physical activity, and possible confounders are also evaluated in relation to the estrogen pathways and included in the final evaluation of the association between estrogen metabolism and prostate cancer risk. | | | | |
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FOREWORD

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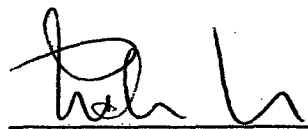
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INTRODUCTION

The primary purpose of this study is to examine estrogen metabolite in relation to prostate cancer etiology. In addition, it also evaluates the possible role of other major risk factors such as diet, lifetime alcohol intake, smoking, lifetime physical activity, and reproductive characteristics in the casual relation with prostate cancer.

In this case-control study, men age 50 to 79 from Erie and Niagara Counties in Western New York with incident, pathologically confirmed cases of prostate cancer are being interviewed. To date we have interviewed 16 African American and 27 Caucasians prostate cancer cases. Controls are randomly selected men, those under age 65 from lists provided by AM-PRO (American-Professional Mailing List Company, Inc.), those are 65 and over from enrollment list of the Health Care Finance Administration. Controls are frequently matched to cases on age, race, and county. Estrogen metabolites are measured in the urine samples. Urine are collected in the morning, after overnight fasting, between 7:00 and 9:00. Additional blood samples are collected, at the same time of urine collection, from all participants to be stored in a biological specimen bank system already developed at the Department of Social and Preventive Medicine, State University of New York of Buffalo, Buffalo, NY. The availability of blood samples stored in the biological specimen bank will offer the opportunity to perform future determinations to test hypotheses which at the present are not yet conceived or for which there are only complex laboratory techniques (i.e., phytoestrogens).

The prostate gland has historically been considered the prototype of an androgen-dependent organ. However, there is evidence that estrogens may induce mitosis of prostatic epithelial cells in many species, including humans. In humans, 16α hydroxyestrone and estriol are biologically significant estrogens, and their biologies can contribute to the overall expression of estrogenic action. The other prominent pathway for metabolism is hydroxylation of C-2, producing 2-hydroxyestrone, which has virtually no estrogenic activity, except for some of the central activities of estradiol, such as the regulation of pituitary hormone release.

In humans, estrogen metabolism has been primarily studied in women in relation to breast cancer risk. Clinical and metabolic studies have shown that some environmental factors, associated with protection/risk for prostate cancer, such as diet, physical activity and obesity are able to induce changes in the estrogen metabolic pathways.

Analytical epidemiological studies on effects of estrogens in relation to prostate cancer risk, in particular serum estrone and estradiol have provided conflicting results. The inconsistency of results may be due to chance, low statistical power (low number of cases and controls), different strategy of control selection and different methodology in specimen collection (i.e., different control of some sources of hormone variability as circadian rhythm or different storage conditions). It may also be that the relevant measure is not the serum level of estrone and estradiol but the estrogen metabolism. A sedentary life-style, an usual diet characterized by high fat and low vegetables, in particular low in cruciferous, and obesity may increase estrogen metabolism towards 16α hydroxylation. This preferential pathway produces a stable hyperestrogenic environment and induces mitosis of prostate epithelial cells. An opposite life-style and a diet rich in vegetables may induce prevalent 2-hydroxylation and produce opposite effects.

The study hypothesis is that an estrogen metabolism more oriented toward 2-hydroxylation, than toward 16α -hydroxylation is protective for prostate cancer.

BODY OF REPORT

During the first budget year, study protocols were developed and submitted to 5 area hospitals for review by their Institutional Review Boards. As of this date, we have confirmed written approval from all 5 hospitals.

In the Statement of Work (Task 1), we estimated that we would only require 3 months to complete the approval process. This task, however, took almost a full year. We encountered some difficulties in obtaining approval, in particular, from one of the area hospitals. These difficulties however, have been resolved.

During this period, we collaborated with a few physicians and have finalized the questions on risk factors for prostate cancer as well as preparing letters for both cases and controls and developing strategies for recruitment. In addition, we tried to expand the base of prostate cancer case recruitment by including all private practices in Erie and Niagara Counties. After six months of contacts and calls and visits, we have several offices that are collaborating with us. A specific form for a standard staging of prostate cancer across all hospitals and private practices has been developed by our group (see appendix section). We finalized all arrangements for interviewing and have begun interviewing cases and controls as listed in the Statement of Work (Task 2). We are continuing to enroll controls as part of the already existing case-control studies. We hired a Project Coordinator, a part time data manager, a part time administrative assistant, and two part time people to interview and recruit. We have developed a poster and brochures to be distributed to doctors' offices to support our recruitment efforts.

After developing letters and making minor revisions, we began to actively recruit cases and controls in April, 1999, in accordance with the Statement of Work (Task 2). We began working both with hospital personnel in pathology in which we had IRB approval and with a few private Urologists. After obtaining names of cases with newly diagnosed prostate cancer between the ages of 50 and 79, we mail a request to the physician for permission to interview the patient. Once the approval is received, we send a letter to the patient inviting them to participate. A written consent is obtained from the patient for both the blood draw and for the interview before the actual process takes place. So far, we have identified 122 prostate cancer cases in Erie and Niagara Counties. Of the 122 cases, 16 were not approved by their physician, 41 were not eligible because they started treatment, 13 declined to take part in the study, 3 we were not able to contact by phone or by mail, 1 person died, and 5 were too ill to participate in the study. To date, we have interviewed 43 prostate cancer cases and all have provided blood and urine samples. We have also interviewed and collected blood and urine samples on 185 controls from Erie and Niagara Counties. A PSA determination has been done on all controls to rule out any latent prostate cancer. We have been able to identify 3 prostate cancer cases as a result of the PSA determinations that were done. The PSA results were sent to the participant's primary physician along with a letter to draw attention to the fact that it was outside of the normal range. The primary physician then referred the

participant to a urologist where a biopsy was done to confirm prostate cancer.

Procedures have been finalized for the ongoing maintenance of the biological specimen bank, processing of samples for immediate determinations and for storage, tracking of samples and mapping of the freezer as outlined in the Statement of Work (Task 2). For standardization purposes, all blood is drawn during the same hour of the day (7:00AM – 9:00AM) and is drawn in a fasting state and processed within one hour of the blood draw. The time of the blood draw is recorded for assessment of any variation in blood markers related to the time of the draw.

Training has been provided for all interview staff and has been coordinated to coincide with the activity of on-going case-control studies. Hard copies of the questionnaire were provided, interview techniques for standardization were reviewed and training provided. In accordance with the Statement of Work (Task 2), preparations for the ongoing data entry of the interview, maintenance of files from the computer-assisted interview, and entry of data from the sections of the interview completed by hand by the participant were developed and coincide with procedures used in the existing case-control studies. Several databases have been created. The central database for the prostate cancer study is written in Microsoft Access and contains information on all participants as well as prospected non-participants. Each form that a study participant fills out has a computer equivalent. Each of these databases is housed in the same location for security, backup, and data analysis purposes. These programs were written in Microsoft Access as well. Data validation, analysis, and compatibility (for integration with SPSS) have been written in Visual Basic. Training has also been provided for the identification and staging of prostate cancer cases in area hospitals as well as the private physician offices.

This first year has been very difficult as far as gaining approval from private urologists to have their patients take part in this study. We have met with several urologists in their office to explain the study and to give them a copy of the protocol and some physician approval forms along with brochures and posters. Letters were written regularly as well as weekly calls to the office managers to remind them of the study.

We have contacted a few organizations such as US TOO and A FAMILY AFFAIR. They were extremely helpful and gave some constructive criticism as well as writing an article about the study in their monthly newsletter. We continue to have a good relationship with these support groups.

Several presentations were given to groups of urologist alerting them of the study and inviting them to help by giving and approving names of their patients who would fit the criteria.

In addition, we sought the help of larger groups of physicians. We talked with the Promedius Group and the Catholic IPA. They were also very helpful and sent a letter to

the physicians that were part of their group telling them that they were behind this study and it should be supported.

We have had special memo pads, pens and work sheets made up that are regularly sent to the area urologists along with physician approval forms and some up to date articles that may be of interest to them. We are continually looking for new recruitment strategies and new ways of attracting the attention of the urologists in this area.

Publications and Presentations

At the present time, there are no results or publications coming directly from this grant because we have just begun data collection. However, Dr. Muti has published or has in press research on hormone related cancer using a previously collected data set. In 1999, she presented results regarding hormone activity and cancer risk at the Annual Meeting of the American Association for Cancer Research (1) and the Annual Meeting of the Society for Epidemiologic Research (2). In the first study, we focused on three peripheral indices of androgenic activity (body fat distribution, sebum production, and hirsutism) and breast cancer risk in premenopausal and postmenopausal women. We found that there was a risk associated with abdominal adiposity in premenopausal women and a risk associated with sebum production and hirsutism in postmenopausal women. In the second study on estrogen metabolism and breast cancer risk, we found that in premenopausal women, a higher ratio of 2-hydroxyestrone to 16 α -hydroxyestrone was associated with a 40% reduced risk of breast cancer. In postmenopausal women, no association with the ratio was observed.

Both of these studies have been submitted for publication.

Furthermore, we have in publication a manuscript on estrogen reliability in premenopausal women (Nutrition Metabolism and Cardiovascular Disease, 2000) (please see appendix for the manuscript).

CONCLUSIONS

We have just begun data collection for this grant; therefore, there are no conclusions to report at this time. Interview of participants is underway.

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- 1) Muti P, Bradlow HL, Micheli A, Krogh V, Freudenheim JL, Schünemann HJ, Stanulla M, Yang J, Sepkovic DW, Trevisan M, Berrino F. Estrogen Metabolism and Risk of Breast Cancer: A Prospective Analysis of the 2:16 α -Hydroxyestrone Ratio in Pre- and Postmenopausal Women. Abstract presented at the Society for Epidemiologic Research, Baltimore, MD, June 1999.
- 2) Muti P, Stanulla M, Micheli A, Krogh V, Freudenheim JL, Yang J, Schünemann HJ, Trevisan M, Berrino F. Markers of Insulin Resistance and Sex Steroid Hormone Activity in Relation to Breast Cancer Risk: A Prospective Analysis of Abdominal Adiposity, Sebum Production, and Hirsutism. Abstract presented at the American Association for Cancer Research, Philadelphia, PA, April 1999

APPENDIX

- Appendix 1: **Estrogen Metabolism and Breast Cancer Risk: A Prospective Analysis in Pre- and Postmenopausal Women.**
- Appendix 2: **Markers of Insulin Resistance and Sex Steroid Hormone Activity in Relation to Breast Cancer Risk: A Prospective Analysis of Abdominal Adiposity, Sebum Production, and Hirsutism.**
- Appendix 3: **Why And How It Is Important To Consider Reliability of Urinary Sex Steroid Metabolites In Studies On Hormones In Women**
- Appendix 4: **Prostate Cancer Clinical Information Form**

APPENDIX 1

Estrogen Metabolism and Breast Cancer Risk: a Prospective Analysis in Pre- and Postmenopausal Women (Abstract, American Journal of Epidemiology 1999; 192:S48.)

Muti P, Bradlow HL, Micheli A, Krogh V, Freudenheim JL, Schünemann HJ, Stanulla M, Yang J, Sepkovic DW, Trevisan M, Berrino F.

It has been hypothesized that the path of estradiol metabolism has an impact on breast cancer etiology. Experimental and clinical evidence suggests that 16 α -hydroxylated estrogen metabolites, more biologically strong estrogens, are associated with breast cancer risk, while 2-hydroxylated metabolites with lower estrogenic activity, are more weakly related to this disease. The present study analyzes the association of breast cancer risk with estrogen metabolism, expressed as the ratio of 2-hydroxyestrone to 16 α -hydroxyestrone in a prospective nested case-control study. Between 1987 and 1992, 10,786 women (aged 35-69) were recruited in a prospective study on breast cancer in Italy, the ORDET study. Women with a history of cancer and women on hormone therapy were excluded at baseline. At recruitment, overnight urine was collected from all participants and stored at -80°C. After an average of 5.5 years of follow-up, 144 breast cancer cases and four matched controls for each case were identified among the participants of the cohort.

Among premenopausal women, a higher ratio of 2-hydroxyestrone to a 16 α -hydroxyestrone at baseline was associated with a 40% reduced risk of breast cancer: women in the highest quintile of the ratio had an adjusted relative risk (RR) for breast cancer of 0.58 [95% confidence interval (CI) 0.25-1.34], p for trend 0.04. In postmenopausal women no association with the ratio 2- to 16 α -hydroxyestrone was observed: adjusted RR for the highest tertile of the estrogen metabolite ratio was 1.29 (0.53-3.10), p for trend 0.89.

Results of this prospective nested case-control study support the hypothesis that the estrogen metabolism pathway favoring 2-hydroxylation over 16 α -hydroxylation is associated with a reduced risk of invasive breast cancer risk for premenopausal women.

APPENDIX 2

Markers of Insulin Resistance and Sex Steroid Hormone Activity in Relation to Breast Cancer Risk: A Prospective Analysis of Abdominal Adiposity, Sebum Production, and Hirsutism. (Abstract, Proceedings of the American Association for Cancer Research, March 1999, 211: #1397)

Muti, P, Stanulla M, Micheli A, Krogh V, Freudenheim JL, Yang J, Schünemann HJ, Trevisan M, Berrino.

Objective: Insulin resistance and increased levels of serum steroids have been hypothesized to be relevant etiological factors for breast cancer. Measurements of markers of insulin resistance and elevated serum steroids may identify women at high risk for breast cancer. The present study analyzed the association of breast cancer with markers of insulin resistance and elevated serum sex steroids, abdominal adiposity, increase in sebum production and hirsutism in a case-control study nested in a prospective cohort study.

Methods: Between 1987 and 1992, 10,786 women (aged 35-69) were recruited in a prospective study on breast cancer in Italy, the ORDET study. Women with history of cancer and on hormone therapy were excluded at baseline. At recruitment, abdominal adiposity was calculated from the ratio of waist-to-hip circumferences. Sebum production was measured on the forehead under standardized conditions using a sebumeter. Nine androgen-sensitive body areas were evaluated for hirsutism and a total hirsutism score was computed. After an average of 5.5 years of follow-up, 144 breast cancer cases were identified among the participants of the cohort. For each breast cancer case, four matched controls were randomly chosen from members of the cohort who did not develop breast cancer during the follow-up period.

Results: Waist-to-hip ratio was associated with breast cancer in premenopausal women: age and body mass index (BMI) adjusted relative risk (RR) for the highest tertile of waist-to-hip ratio was 2.2 [95% Confidence Interval (CI) 1.04-4.75], p for trend 0.03. In the analysis conducted within strata of BMI, the effect of waist-to-hip ratio was confined to the group of thinner women: RR for the highest tertile of waist-to-hip ratio was 3.4 (95% CI 1.2-9.5).

Sebum production and hirsutism were associated with breast cancer among postmenopausal women. Age and BMI adjusted RRs for the upper tertiles were 2.2 (95%CI 1.1-4.6), p for trend 0.01, and 2.3 (95% CI 1.1-4.9), p for trend 0.03, for sebum and hirsutism, respectively.

Conclusion: These results add evidence for a role of hormones and metabolic alterations in breast cancer etiology and for different relations of these risk factors with breast cancer in premenopausal and post menopausal women.

APPENDIX 3

Manuscript in publication

PROPRIETARY

Why And How It Is Important To Consider Reliability Of Urinary Sex Steroid Metabolites In Studies On Hormones In Women

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Running Title: Reliability of estrone and pregnandiol-3-glucuronide

Abstract

Although numerous investigations have evaluated the association between urinary hormone levels and chronic diseases such as breast cancer and coronary heart disease, there is limited data about the reliability of urinary measurements, particularly among premenopausal women. Over a six-month period, levels of estrone-3-glucuronide and pregnandiol-3-glucuronide were measured in both morning spot and overnight urine samples from seven healthy premenopausal women (ages 33-46). During the six-month period, each subject provided one morning spot urine sample and one overnight urine sample per menstrual cycle on the same day of her menstrual cycle. All samples collected over the six-month period were taken out of the freezer simultaneously and sent in the same parcel on dry ice to the laboratory for the hormonal determinations. All samples from each person were assayed simultaneously in the same run, by the same laboratory technician in a blind fashion. The intraclass correlation coefficients (ICC) for estrone-3-glucuronide for the morning spot and overnight urine samples were 0.78 and 0.46, respectively. For pregnandiol-3-glucuronide, the morning spot and overnight urine ICCs were 0.75 and 0.64, respectively. These data suggest that among healthy premenopausal women estrone-3-glucuronide and pregnandiol-3-glucuronide determinations in morning spot urine showed good reliability, and that morning spot urine samples might represent a efficient alternative to the more complex overnight urine collection for epidemiological studies to evaluate urinary hormonal profiles.

Introduction

The usefulness of measurement in clinical and epidemiological research depends on the extent to which a researcher can rely on data as accurate and meaningful indicators of an exposure or presence of disease. Thus, reliability or the extent to which a measurement is consistent and free from error is a very relevant aspect of measurement to be addressed in the planning of research protocols. Reliability can be conceptualized as predictability: for instance, a reliable instrument is one that will perform with predictable consistency under specific condition. Theoretically, it is possible to look at any "observed score" (X) as a function of two components: a "true score" (T) and an "error component" (E).

In analytical determinations of biomarkers, the sources of "error component" are two: technical variability (or the variability related to the act of measuring), and the biological variability (or the variability due to the biological characteristics of a specific biomarker, e.g., circadian variation of several serum biomarkers).

The purpose of the present report is to describe "why and how" is important, in planning clinical and epidemiological research on hormone related disease, to consider the "error component" due to hormone biological variability. In particular, it reports data on size and effect of this error in studies including estrone-3-glucuronide and pregnandiol-3-glucuronide determination in urine. Many epidemiological studies have investigated the association between endogenous sex steroid levels and chronic diseases such as breast cancer and coronary heart disease (1-3). Hormone levels in serum are thought to be the most accurate indicator of hormone secretion patterns, whereas hormone levels determined from urinary samples reflect the end product of a complex path of secretion, metabolism and excretion (4). Some case-control studies used urinary estrogens to determine the endocrine patterns associated with the risk of breast cancer (5-15),

with inconsistent results. These studies often relied on a single measurement of urinary hormone level, although there was limited information about the reliability of these parameters, particularly for premenopausal women. Large intra-individual variability may lead to misclassification of subjects and attenuated risk ratios (16-18). The present study was conducted to evaluate the reliability of measurements of two urinary glucosiduronate metabolites of serum steroids: estrone-3-glucuronide, and pregnandiol-3-glucuronide in morning spot and overnight urine samples.

Estrone-3-glucuronide is one of the main products of the serum estrogens inactivation and conjugation and provides a useful measure of ovarian function (19-20). Furthermore, it correlates with serum level of serum estradiol, the main serum estrogen in premenopausal women (4). Pregnanediol-3-glucuronide is the main product of serum progesterone. Measurements of pregnandiol-3-glucuronide in urine have been found to correlate highly with serum progesterone levels, which makes it an important biomarker of ovarian luteal function (21). The present study included healthy premenopausal women who provided monthly urine samples during a six-month period. The subjects were in different phases of the menstrual cycle, but each woman provided urine samples for the same day of her menstrual cycle across the six months. The study design controlled for several potential sources of biological (i.e., circadian rhythm) and laboratory variability.

Materials and Methods

Study Methods

The present study was conducted as a pilot investigation for a series of case-control studies of life-time alcohol intake and chronic disease among persons living in Erie and Niagara Counties in New York state. The protocol was approved by the Internal Review Board of the State University of New York at Buffalo. Participants of the present study were seven premenopausal women who were between 33 and 46 years of age. The subjects were non-smokers, in generally good health and reported regular menstrual cycles, with lengths of 24 to 31 days. None of the subjects were pregnant, breastfeeding or on hormonal therapy during the two months prior to the study or during the study period. No change in body weight was reported by the participants during the six months of the study.

Specimen Collection

The protocol required each subject to provide one morning spot and one overnight urine sample per menstrual cycle during the six-month period. Five participants missed one sample during the study period and one subject missed two samples. Three women collected their urine samples on the same "numerical" day of the follicular phase (days six to eight counting the first day of the menstrual bleeding as the first day of menstrual cycle), two women collected the samples on the same "numerical" day of the luteal phase (days 20 to 24), and two women collected urine in the time between the late luteal and the early follicular phases (days 27 to three) as a result of early menstrual periods. The overnight urine collection protocol called for discarding of the last void before going to bed and collection of all urine voided during the night including the first void of the morning. Overnight urine samples were kept at room temperature. Completeness of the overnight urine collection was determined by asking the participants about

the quantity of urine lost during the collection. All participants reported accurate overnight urine collection. Morning spot urine samples were collected when participants delivered the overnight urine samples between 8:00 and 10:00 A.M. to the Center for Preventive Medicine in the Department of Social and Preventive Medicine at State University of New York at Buffalo, NY. No preservatives were added to the urine. At the Center, samples were processed soon after the delivery of the overnight sample and the collection of the morning spot sample. The urine samples were filtered, and 1 mL aliquots were stored at -70°C . All samples collected over the six-month period were taken out of the freezer simultaneously and sent in the same parcel on dry ice to the laboratory for the hormonal determinations.

Laboratory methods

All samples from each subject were assayed simultaneously in the same run, by the same laboratory technician, and in a blind fashion. Estrone-3-glucuronide was measured using 100 μL of urine mixed with 1.9 mL of assay buffer (0.05 M phosphate/EDTA buffer with 0.1% sodium azide and 0.1% bovine serum albumine) to give a dilution of 1:20 (v/v). Aliquots were assayed in duplicate. Standard solutions were prepared containing 2,000, 1,000, 500, 250, 125, 62.5, 32.25 and 15.125 pg of estrone-3-glucuronide/0.1 mL of assay buffer. Standards and samples were mixed with tritiated estrone (100 μL containing approximately 22,000 dpm/31 pg) and with antiserum (suitably diluted in 100 μL of buffer). The total volume of the incubation mixture was brought to 500 μL with addition of buffer, thoroughly mixed, and incubated at 4°C (18h and 4h or the first and the second experience, respectively). The antibody-bound and free steroids were separated with 0.5 mL of dextran-coated charcoal (2.5 g of charcoal and 0.25 g of dextran T70 per liter) maintained in suspension with a magnetic stirrer. After equilibration for 15 minutes at

4°C the mixture was centrifuged for 15 minutes at 10°C at 2,000 g. The supernatant was decanted into a polypropylene vial containing 4 mL of scintillation fluid (Pico-Fluor 40 from Packard). The solutions were mixed and the absolute amount of radioactivity was determined by counting each vial for 10 minutes. The unknown concentrations of samples were derived from the standard curve by automatic program based on 4PL algorithm and the values multiplied by 0.2 to give the results in ng/mL urine (to convert the ng/mL in SI units – nmol/L – multiplied by 2.24).

Pregnandiol-3-glucuronide was measured using 100 µL of urine mixed with 1.9 mL of assay buffer (0.05 M phosphate/EDTA buffer with 0.1% sodium azide and 0.1% bovine serum albumin) to give a dilution of 1:400 (v/v). The same dilutions were used as described above for the estrone-3-glucuronide determinations. Aliquots (50 µL and 100 µL for luteal and follicular phase, respectively) were assayed in duplicate. Standard solutions were prepared containing 2,000, 1,000, 500, 250, 125, 62.5, 32.25 and 15.125 pg of 5β-pregnanediol-3α-glucuronide/0.1 mL of assay buffer. Standards and samples were mixed with tritiated 20α-hydroxyprogesterone (100 µL containing approx. 11,000 dpm/53 pg) and with antiserum (suitably diluted in 100 µL of buffer). The total volume of the incubation mixture was brought to 500 µL with additional buffer, thoroughly mixed, and incubated at 4°C (18h and 4h or the first and the second experience, respectively). The antibody-bound and free steroids were separated with 0.5 mL of dextran-coated charcoal (2.5 g of charcoal and 0.25 g of Dextran T70 per liter) maintained in suspension by a magnetic stirrer. After equilibration for 15 minutes at 4°C the mixture was centrifuged (15 minutes at 10°C at 2000 g) and the supernatant decanted into a polypropylene vial containing 4 mL of scintillation fluid (Pico-Fluor 40 from Packard). The solutions were

mixed and the absolute amount of radioactivity determined by counting each vial for 10 minutes. The unknown concentrations were derived from the standard curve by automatic program based on 4PL algorithm and the values multiplied by 8 and 4 for luteal and follicular samples, respectively to give the results in ng/mL urine (to convert the ng/mL in SI units – nmol/L – multiplied by 2.24).

The intra-assay analytical variability, expressed as a coefficient of variation percentage (CV %), was measured during the study by using commercial lyophilized control serum at different concentration levels (Lyphochek; Bio-Rad, Milan, Italy). Quality control samples were added to unknown samples at the beginning, middle and at the end of each run to control for drifts in the assaying procedures. No drift of the control values was observed, indicating no obvious systematic error during the measurements. The results are presented in table 1.

Statistical Methods

Means and standard deviations for urine hormone determinations were computed for each subject. Estimates of variance components (within and between variance) and the intraclass correlation coefficients (ICC) were calculated using the method described by Fleiss (22). To indicate the lower limit of uncertainty of the degree of reliability, the lower limit of the 95% confidence interval of the intraclass correlation coefficients was calculated in addition to the point estimate (22). The minimum number of replicate measurements which would be necessary to correctly estimate the hormone level after setting a desired reliability level of 0.90 was computed using the Spearman-Brown formula (22). The degree of linear association between urinary hormone metabolite measured in the morning spot and in the overnight urine samples was analyzed with the Pearson correlation coefficient using the complete data across all subjects and all replicates. The software package used for descriptive data and the correlations was the

Statistical Package for Social Sciences for Windows (version 7) (23).

RESULTS

For each of the seven volunteers, means and standard deviations for urinary estrone-3-glucuronide and pregnandiol-3-glucuronide measured in morning spot and overnight collections across the replicates are shown in tables 2 and 3, respectively. Hormone metabolites were generally more concentrated in the morning spot urine than in the overnight urine collection (24). Specimens for subjects 1 and 5 were collected during the cycle luteal phase and showed a high urinary concentration of the steroid metabolites. With, as expected, pregnandiol presenting the highest levels. Subjects 2 and 3 were drawn in between the late luteal and the early follicular phase a period during which progesterone changes from the highest to the lowest serum level in the menstrual cycle. Concentration of pregnandiol in urine follows the changes in concentration of its serum precursor. Consistent with this pattern, among the seven subjects, subjects 2 and 3 showed intermediate concentration of pregnandiol glucuronide.

Table 4 shows the estimates of variance components, the reliability coefficients, their lower 95% confidence limit and the number of replicates necessary for reliable estimation of estrone-3-glucuronide and pregnandiol-3-glucuronide measurements in morning spot and overnight urine samples. For estrone and pregnandiol, and for both morning spot and overnight urine collections, between-subject variance was larger than within-subject variance indicating good reliabilities of their measurements.

A higher coefficient was observed for estrone and pregnandiol in the morning spot than in the overnight urine collection. In particular, estrone-3-glucuronide showed a large difference in the coefficients between morning spot and overnight urine collection with ICCs of 0.78 and 0.47, respectively. Using the Spearman-Brown formula, we estimated that three replicates would be required for both urinary metabolites using morning spot samples, and that ten and five replicates

would be required for estrone and pregnandiol determinations in overnight urine samples.

Finally, estrone-3-glucuronide and pregnandiol-3-glucuronide concentrations in the morning spot urine showed good correlation with the concentration in the overnight urine collection (Pearson's $r = 0.7$ and 0.8 , respectively).

DISCUSSION

In the last twenty years, urine has been considered as a relevant specimen to be collected in population-based studies of breast cancer and other chronic disease. There is now renewed interest in urine samples as a result of large epidemiological studies that collect and store specimens in biological specimen banks (25). Urine samples have the advantage of being noninvasive and easy to collect. Furthermore, new laboratory methods have been adapted to urine and formatted into economical assays particularly suitable for epidemiological and clinical studies on large number of subjects (26). However, information of intra-individual variability of urinary metabolites is still limited, particularly for hormone metabolites in premenopausal women. To satisfactory test for an association between physiologic variables and risk of diseases within a population, individual subjects must first be adequately characterized. This task is particularly difficult for hormone metabolites in young women due to circadian and monthly fluctuation of hormone precursors in serum, as well as other factors contributing to the hormone metabolism and excretion such as cigarette smoking or medication intake.

The urinary steroid metabolite measurements included in this study showed moderate to good reliability with intraclass correlation coefficients (ICC) ranging from 0.47 (estrone – overnight) to 0.78 (estrone -- morning spot). As expected, measures made during the luteal phase of the menstrual cycle had the highest level of urinary pregnandiol.

Measurements of the urinary metabolites of progesterone and especially estrogens in the morning spot urine were found to be more reliable than in the overnight urine samples. A possible explanation of these results might be that the concentration of the metabolites tended to be higher in the morning spot than in the overnight urine samples with a resulting lower intra-assay variability effect in the morning spot measurements. The observation of a higher level of

urinary metabolites in the morning spot compared to the overnight sample has been reported by others (24). It is not clear why this was observed, however, it may be related to the circadian rhythm of hormones. Panico and colleagues (27) reported more elevated levels of estrogen and progesterones in the morning hours.

The good correlation between the levels of estrone and pregnandiol in morning spot and the overnight urine samples might suggest the use of the morning spot urine as a possible alternative to the more complex overnight urine collection in epidemiological studies to evaluate urinary hormonal profiles.

The small sample size and the missed collections at one time point for five women are the major study limitations. However, to our knowledge, this is the first investigation in which the reliability of urinary hormone metabolites over a short period of time was evaluated while controlling for several sources of biological and methodological variability using strict inclusion criteria and highly standardized urine collection procedures. These procedures included the urine collection between 8:00 and 10:00 A.M., restriction of urine collection in the same menstrual phase of the cycle, exclusion of participants taking hormone therapy, and assaying all samples from the same person at the same time to eliminate the effect of the inter-assay variability.

This study was conducted to assess the consistency of two hormone metabolite determinations from urine samples in a group of seven healthy premenopausal women over a six month period. By design, variation related to circadian rhythm and inter-assay laboratory variability was minimized. Under these conditions, estrone-3-glucuronide and pregnandiol-3-glucuronide levels in urine morning spot showed good reliability.

In order to better characterize individual risk profile, replication of this study with a

larger sample evaluating at the same time consistency and predictability of determinations for precursors and metabolites of urinary hormone metabolites would be important such that a better understanding can be achieved of their role in the etiology of breast cancer.

Acknowledgments

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Table 1. Analytical Variability of Hormone Assays

| Hormone level (mean level of control sera) | Intra-assay variability | |
|---|-----------------------------|---------|
| | number of determinations | CV (%)* |
| Estrone-3-Glucuronide (ng/mL) | | |
| 17.59 | 4 | 7.7 |
| 34.61 | 4 | 5.9 |
| Pregnandiol-3-Glucuronide | | |
| 634.45 | 4 | 18.3 |
| 2185.29 | 4 | 9.9 |
| 9168.58 | 4 | 11.9 |
| 19521.29 | 4 | 10.1 |

* coefficient of variation = (SD/mean) x 100

Table 2. Mean (\pm SD) of Estrone-3-Glucuronide from Morning Spot and Overnight Urine Collection for Each of the Seven Study Participants

| Subject | Number of Samples | Morning Spot Urine | Overnight Urine |
|---------|----------------------|--|--|
| | | Sample: Mean (SD) Level of Estrone (ng/mL) | Sample: Mean (SD) Level of Estrone (ng/mL) |
| 1 | 5 | 76.22 (24.7) | 58.62 (32.6) |
| 2 | 4 | 31.55 (13.3) | 11.19 (5.2) |
| 3 | 5 | 25.96 (6.7) | 10.88 (4.1) |
| 4 | 6 | 16.29 (11.1) | 23.37 (10.4) |
| 5 | 5 | 44.98 (7.8) | 24.16 (6.1) |
| 6 | 5 | 13.21 (5.9) | 23.42 (17.0) |
| 7 | 6 | 11.48 (6.8) | 21.00 (7.9) |

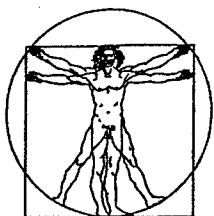
Table 3. Mean (\pm SD) of Pregnandiol-3-Glucuronide from Morning Spot and Overnight Urine Collection for Each of the Seven Study Participants

| Subject | Number of Samples | Morning Spot Urine | Overnight Urine |
|---------|-------------------|--|--|
| | | Sample: Mean (SD) Level of Pregnandiol (ng/mL) | Sample: Mean (SD) Level of Pregnandiol (ng/mL) |
| 1 | 5 | 8901.6 (2241.2) | 6681.8 (3192.8) |
| 2 | 4 | 4635.5 (3102.2) | 1981.3 (2179.8) |
| 3 | 5 | 2847.4 (2308.3) | 1309.2 (919.7) |
| 4 | 6 | 809.3 (543.2) | 903.8 (436.2) |
| 5 | 5 | 4099.6 (893.7) | 2323.4 (680.9) |
| 6 | 5 | 1042.5 (369.8) | 1627.6 (1321.0) |
| 7 | 6 | 772.1 (733.1) | 1307.3 (535.1) |

Table 4. Estimate of Variance Components, Intraclass Correlation Coefficient (ICC) and Lower Limit of the 95% Confidence Interval (95% CI) for Estrone-3-Glucuronide and Pregnandiol-3-Glucuronide in Morning Spot and Overnight Urine Samples

| Hormones | Estimate of Variance Components | ICC | 95% CI (Lower-bound) | Minimum Number of Replicates | | | |
|----------------------------------|---------------------------------|-------|----------------------|------------------------------|--|--|--|
| Estrone-3-Glucuronide | | | | | | | |
| <i>Morning Spot Samples</i> | | | | | | | |
| Between Subjects | 17896.4 | 0.783 | 0.579 | 3 | | | |
| Within Subjects | 4415.0 | | | | | | |
| <i>Overnight Urine Samples</i> | | | | | | | |
| Between Subjects | 7539.1 | 0.468 | 0.200 | 10 | | | |
| Within Subjects | 6592.0 | | | | | | |
| Pregnandiol-3-Glucuronide | | | | | | | |
| <i>Morning Spot Samples</i> | | | | | | | |
| Between Subjects | 268182570.1 | 0.752 | 0.531 | 3 | | | |
| Within Subjects | 79190011.0 | | | | | | |
| <i>Overnight Urine Samples</i> | | | | | | | |
| Between Subjects | 121087501.9 | 0.645 | 0.388 | 5 | | | |
| Within Subjects | 56615388.5 | | | | | | |

APPENDIX 4



PROMEN STUDY

Prostate Cancer Clinical Information Form

ID # | _____ |
Name | _____ |

DOB | _____ |

The present form and instructions are intended to be used to collect data for an epidemiologic case-control study. Therefore they cannot be directly applied to a clinical setting. Please contact Dr. Paola Muti at the Department of Social and Preventive Medicine, School of Medicine and Biomedical Sciences, SUNY at Buffalo, N.Y. for information on the present instruments. This form is to be completed by trained PROMEN study personnel on the basis of clinical chart information.

Date of diagnosis

____/____/____
Month Day Yr

Date of first hospital admission

____/____/____ | _____ |
Month Day Yr hospital

Date of previous diagnosis of prostate cancer

____/____/____ | _____ |
Month Day Yr hospital

In situ

____/____/____ | _____ |
Month Day Yr hospital

Previous or synchronous cancer:

Site.....Hist.....

Date at Diagnosis ____/____/____
Month Day Yr
| _____ |
hospital

PRESENTATION AT DIAGNOSIS

- N.S. Modality ☐
- Symptoms: Urinary symptoms ☐ Haematuria / Haemospemia ☐
Pain from bone metastasis ☐ Pain in the prostatic region ☐
- Exams not related with symptoms ☐ Rectal examination ☐ Echography ☐ PSA ☐
Discover of bone metastasis by X ray in absence of a clinical suspect ☐
- Incidental histological diagnosis (with negative imaging and rectal examination) ☐
- Occult carcinoma (Epicritic diagnosis with negative local diagnostic exams) ☐

REPORT HISTOLOGY

Histology from needle biopsy ☐

Histology from radical prostatectomy specimen ☐

Histo/cytology not performed ☐

Date of histo/cytology ____/____/____
day month year

Histological Grading I (well)

II (moderately)

III (poorly differentiated)

Gleason score 2 3 4

5 6 7

8 9 10

Gleason pattern 1-2

3

4 5

1-2

3

4 5

STAGE reported in clinical/pathological notes T__N__M__(pT and pN if available ☐☐)

A1 ☐ A2 ☐ B1 ☐ B2 ☐ C1 ☐ C2 ☐ D1 ☐ D2 ☐ N.S. ☐

Reconstructed stage from clinical and pathological notes:

A(TI) : INCIDENTAL TUMOR

a: histologic finding in 5% or less of tissue resected; b: histologic finding in more than 5% of tissue resected; c: tumor identified by needle biopsy (e.g. because elevated PSA)

B(T2): CLINICAL OR IMAGING EVIDENCE, BUT TUMOR CONFINED WITHIN PROSTATE

< 15 mm ☐ > 15 mm ☐ 2 lobes ☐ N.S. ☐

C(T3): TUMOR EXTENDS THROUGH PROSTATE, INVADES CAPSULE

Without ☐ or within ☐ invasion of seminal vesicles, or (T4) fixation to other pelvic structures ☐ or N.S. ☐

D (NO): No evidence ☐

(N1): 1 single regional node ≤ 2 cm in its greatest dimension ☐

(N2): More than 1 regional node or one 2-5 cm in greatest dimension or multiple nodes ☐

(largest dimension in all nodes, ≤ 5 cm) ☐

(N3): At least 1 node more than 5 cm in greatest dimension ☐

(N+): N.S. ☐

(Nx): cannot be assessed ☐

MO=none ☐

(M1): Distant metastasis ☐

M1a: Nonregional lymph node (or nodes) ☐

M1b: Bone ☐

M1c: One or more other sites ☐

DIAGNOSTIC EXAMINATIONS FOR STAGING:

| | Extension Through Prostatic capsule | | | | | N.S. |
|------------------------|-------------------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| | Done | Not done | No | Suspect | Yes | |
| Digital rectal exam | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Transrectal ultrasound | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| CT prostate | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| MRI prostate | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Histological exam | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |

| | Nodal and distant metastasis | | | | | N.S. |
|-------------------|------------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| | Done | Not done | Neg | Suspect | Pos | |
| Lymphangiography | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| CT of Lymph nodes | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| CT prostate | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |

MRI of Lymph nodes

| | | | | | | |
|---------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| Bone scintigraphy | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Xray of pelvis | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Xray of spine | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Other skeleton Xray | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Chest Xray or CT | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Abdominal CT | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Lymphadenectomy | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |

Number of examined nodes ☐ Number of metastatic nodes ☐

PSA (before therapy) No ☐ Yes ☐ N.S. ☐ Results _____ ng/dL (normal range _____)

PAP (before therapy) No ☐ Yes ☐ N.S. ☐ Results _____ ng/dL (normal range _____)

THERAPY (PROGRAMMED OR PERFORMED)

- Surgery: N.S. ☐ No ☐ Yes ☐
 - Prostatectomy nos ☐ Nerve-sparing radical prostatectomy ☐ Not Nerve-sparing radical prostatectomy ☐
 - Not specified surgery ☐ TURP ☐ Other ☐
 - Radiotherapy: N.S. ☐ No ☐ Yes ☐ High energy ☐ Cobaltum ☐ Interstitial ☐ N.S. ☐
On metastasis ☐ Systemic ☐
 - Endocrine Therapy: N.S. ☐ No ☐ Yes ☐ If yes:
 - 1) Castration ☐ 2) Estrogens ☐ 3) Antiandrogens ☐ 4) GnRH analog ☐
5) Peripheral inhibitors of hormone activity (flutamide) ☐ 6) Other or N.S. ☐
- Please indicate the therapy combination (e.g., only 3 and then 3+4) |__| |__|
- Chemotherapy: No ☐ Yes ☐ N.S. ☐
 - It has been decided not to perform any treatment ☐
 - No therapy reported ☐
 - Brachytherapy ☐



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5017

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

26 Nov 02

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. PINEHART
Deputy Chief of Staff for
Information Management

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